

Antimicrobial Susceptibility of *Euphorbia tirucalli* Extracts and the Effect of Latex on Growth and Viability of Mouse Breast Cancer Cells EMT-6

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Summary: *Euphorbia tirucalli* is a plant presents in many countries which also known as Aveloz. This tree used in traditional medicine for treatment different types of disease. In this research we tested the effects of aveloz latex, stem and leaves extracts on different gram positive and gram negative bacteria, and its effects on some types of fungi, also we tested the aveloz effects on mouse breast cancer cells. So we did extraction for leaves and stems of aveloz by methanol and prepared the latex directly from the tree by aseptic technique. Then we prepared different dilutions from the 3 products, then we prepared the inoculums, culture and sensitivity techniques were be performed, tissue culture for mouse breast cancer cells and latex were performed to study its anti-cancer effects. Results showed us there were effects of the stem and leaves extracts on some types of bacteria where the latex appeared a best sensitivity on some fungi species. There was an interesting effect of latex on cancer cells. Aveloz products can be used in producing materials to prepare medical products may be help in treating different diseases.

Key words: *Euphorbiaceae*; *Euphorbia tirucalli*; Aveloz; Latex; EBV (Epstein bar Virus); CFU-GM (colony forming unit- Granulocyte Monocyte)

I. Introduction

Euphorbia tirucalli, a garden plant from Africa, belongs to the family Euphorbiaceae. Other names used include *Tirucalia indica*, *Tithymalus tirucalli*, and *Euphorbia viminalis*. Common names used to for this plant include pencil tree, aveloz. All parts of the tree secrete a white milky fluid referred to as latex (1). The latex of Aveloz contains terpenes including phorbol esters and ingenol esters which cause severe irritation upon direct exposure. It contains fatty acid fractions consist of palmitic and linoleic acids, phytosterol consist of sitosterol, stigmasterol, campesterol and cholesterol, and also contain cyanidin glycoside (2). The latex, stem and leaves of Aveloz tree are used in traditional medicine in many countries to treat several diseases, particularly skin diseases (3).

Therefore, we were tested the stem, leaves extracts and latex, on several multidrug resistant microorganisms. Several gram positive and gram negative bacteria, yeasts and molds were obtained and cultured. Facultative gram positive cocci such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *micrococcus lutens*, *vancomycin resistance enterococcus*, *enterococcus faecalis*, and *MRSA*. Gram negative bacteria such as *Pseudomonas aeruginosa*, *E. coli*, *K. pneumoniae* *Proteus mirabilis*, *Shigella Sonni*, *Shigella flaxiniri*, *Pseudomonas MDR*, and *Actinobacter MDR*. Fungi which include *Candida sp*, *Aspergillus spp*, *Fuzarium*, and *Rhizopus*. Antimicrobial susceptibility was performed following the recommendations of CLSI. In addition, the anti tumor properties of the latex were tested in-vitro on the breast cancer cells EMT-6 cells.

II. Materials and Methods

1.1 Aveloz

Aveloz latex was collected following aseptic techniques directly from the euphorbia tree in a sterile tube. The latex was then placed in a cooler and transferred immediately to the laboratory for proper storage and testing. Aveloz was also extracted from the plant's stem and leaves separately: Pieces from the stem and leaves were individually mixed in a blender and the homogenate was placed in a flask with 200 ml methanol for 24 hours. The liquid was then filtered through several layers of sterile gauze and then centrifuged at 5000xg for 15 minutes. The supernatant was then carefully removed and placed in a white glass plate to evaporate the alcohol. The remaining fluid was then collected and placed in a sterile tube to be tested.

1.2 Microorganisms

ATCC strains of Gram positive, gram negative bacteria and fungus were obtained from the stocks at the laboratory of the Master's Program at Birzeit University. The antimicrobial and anti fungal characteristics of

the aveloz latex, leaves and stem extracts were evaluated on Mueller-Hinton agar following the recommendations of CLSI. The optimal dilution was determined by applying several dilutions of the aveloz to filter paper disks placed on the surface of agar plates seeded with the organism to be tested.

1.3 Effects of Aveloz Latex on Mouse Breast Cancer Cells TEM-6

The mouse breast cancer cells EMT-6 were seeded in 24 wells plates. A total of 1×10^4 cells were seeded in each well and the plate was incubated for 24 hours. The medium was then removed, the cells washed with HBSS and the aveloz latex was applied in triplicate at different dilutions. RPMI-1640 medium supplemented with 10% FBS was added to three wells and used as positive control. After 48 hours of incubation, the medium from each well was removed and placed in 15 ml conical centrifuge tube. Trypsin EDTA was added to each well (200 ul) and the plate was incubated at 37° C for 5 minutes. RPMI-1640 was then added to each well (200 ul) to stop the action of the trypsin and the contents of each well were placed in the respective conical centrifuge tube. After centrifugation for 7 minutes at 700xg, the supernatant was removed and the cells were suspended in 500 ul of RPMI-1640 medium. Total and viability counts were then performed and recorded. The distribution of the different aveloz latex dilutions in the 24 well plates as well as the positive control wells is shown in (Table 1).

Tube	Tubes numbers					
	1	2	3	4	5	6
A	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
B	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
C	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
D	c control	c control	c control	co control	c control	control

Table 1. Distribution of the aveloz latex dilutions in the wells and the positive control wells.

1.3.2 Counting of Cells and Estimation of Viability

The cells (200 ul) were mixed with HBSS (300 ul) and trypan blue stain (500 ul). The tube was briefly vortexed and allowed to stand at room temperature for 5 minutes. A hemocytometer chamber was then properly filled with the stained cells. Four corner squares were then counted. The total number of cells was calculated according to the following formula:

$$\text{Total number of cells} = \text{Average number / square} \times \text{dilution factor} \times 10^4$$

Viability was determined according to the following formula:

$$\text{Viability} = \frac{\text{Total number of cells} - \text{blue or dead cells}}{\text{Total number of cell}}$$

1.4 Inoculums Preparations

The bacteria was inoculated on blood agar plates and incubated for 18 to 24 hours, while fungus was inoculated on Sabaraud Dextrose agar until growth occurs on room temperature. One or two colonies were then removed and a suspension equivalent to 0.5 MacFarland was prepared. A sterile cotton swab was then dipped in the suspension, the excess fluid removed by gently rotating the swab against the inside of the tube containing the suspension. The swab was then used to inoculate the surface of Mueller-Hinton agar plates. Sterile filter paper discs were then aseptically placed on the surface of the agar plates and 5 ul of each aveloz dilution was placed in duplicate to the filter paper. The plates were incubated for 18 to 24 hours in a non-CO2 incubator; the zone diameters were accurately measured using Vernier caliper and recorded.

Several strains of ATCC strains of Gram positive and negative bacteria were used including: Gram –ve bacteria: *Proteus mirabilis*, *Shigella Sonni*, *shigella flaxiniri*, *E. coli*, *Pseudomonas MDR*, *Pseudomonas earugenoza*, *Klebseilla Pneumonia*, *Actinobacter MDR*. Gram + bacteria: *Staphylococcus aureus*, *Staphylococcus epidermides*, *micrococcus lutens*, *vancomycin resistance enterococcus*, *enterococcus faecalis*, *MRSA*. Fungus isolates: *Aspergillus*, *Rhizopus*, *Fuzarium*, *Yeast and Candida species And Mold*.

III. Results

2.1 Effects of Aveloz Latex on Mouse Breast Cancer Cells (EMT-6)

EMT-6 cells (1×10^4) taken during the logarithmic phase of growth were placed in each well of a 24 well plate in triplicate. Different dilutions of the aveloz were added to the wells, and the plates were incubated for 24 hours. The results obtained are summarized in (Table 2). There were complete lyses of the cell up to a

dilution of 1:100. The viability was drastically reduced to a 22% at 1:1000 dilutions. At lower dilution the cell counts and viability were comparable to the control (fig. 1).

cancer cells	Aveloz latex concentrations						
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	0(control)
No of viable cells/ml	0	0	3.0X10 ⁴	66 X10 ⁴	88.8X10 ⁴	93.7X10 ⁴	144.1X 10 ⁴
No of dead cells/ml	1x10 ⁴	1x10 ⁴	10.6 X10 ⁴	3.8 X10 ⁴	5 X 10 ⁴	1.9 X 10 ⁴	1.9 X 10 ⁴
Total no. of cells/ml	1x10 ⁴	1x10 ⁴	13.6 X10 ⁴	69.8X10 ⁴	93.8 X 10 ⁴	95.6 X 10 ⁴	146 X 10 ⁴
% of viability	0%	0%	22%	94%	95%	98%	99%

Table 2. Results of aveloz latex effects on mouse breast cancer cells.

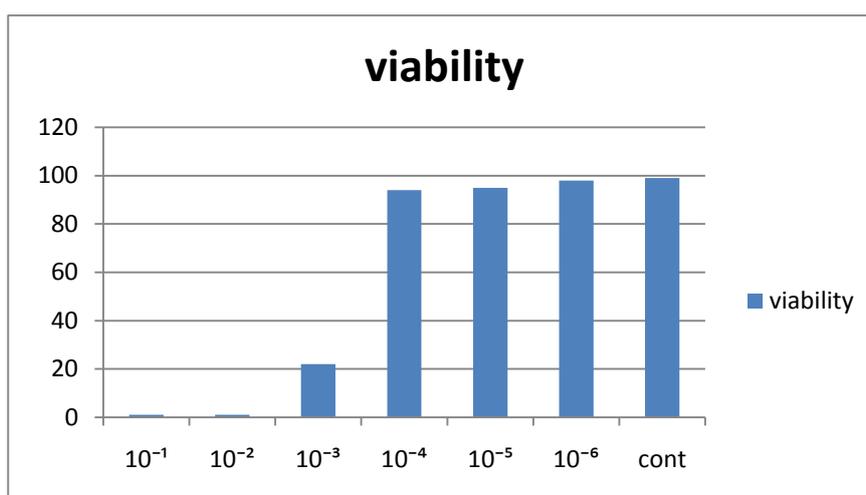


Fig.1 viability (%) of cells at different dilutions (10⁻¹ – 10⁻⁶) of Aveloz, control without aveloz.

2.2 Effects of Aveloz Latex Stem and Leaves Extracts on Bacteria

Aveloz latex was tested on different strains of gram positive and gram negative bacteria. Different dilutions were added onto sterile filter papers placed on a lawn of bacteria spread on a Mueller Hinton agar following the recommendations of CLSI. The results obtained indicate that the aveloz latex had no antimicrobial activity. There was growth up to the edge of the filter paper discs with all dilutions used including a pure extract. Extracts from the stem and leaves demonstrated anti-bacterial activity for both gram positive and gram negative bacteria. Multidrug resistant non-typhoid Salmonella species and Pseudomonas aeruginosa were resistant to all dilutions including the original extract. The zones of inhibition are summarized in (Table 3).

Microbial species	Aveloz latex concentrations				stem extracts concentrations				leaves extracts concentrations			
	Orig conc	1:1	1:2	1:4	Orig conc	1:1	1:2	1:4	Orig conc	1:1	1:2	1:4
<i>Staphylococcus aureus</i>	0	0	0	0	24	16	12	0	14	12	6	0
<i>Staphylococcus epidermidis</i>	0	0	0	0	32	28	20	0	32	28	20	0
<i>Micrococcus luteus</i>	0	0	0	0	28	20	14	12	28	22	10	0
<i>MRSA</i>	0	0	0	0	17	14	0	0	24	18	14	0
<i>Enterococcus Faecalis</i>	0	0	0	0	14	14	0	0	24	18	10	0
<i>Vancomycin resistance enterococcus</i>	0	0	0	0	22	14	6	0	24	20	14	0
<i>Proteus mirabilis</i>	0	0	0	0	20	16	14	0	20	16	14	0
<i>Shigella sonnei</i>	0	0	0	0	22	0	0	0	0	0	0	0
<i>Shigella flexneri</i>	0	0	0	0	30	28	10	0	30	20	10	0
<i>Salmonella</i>	0	0	0	0	0	0	0	0	0	0	0	0
<i>Acinetobacter baumannii</i>	0	0	0	0	20	18	14	0	24	20	8	0
<i>E. coli</i>	0	0	0	0	30	20	16	0	30	20	10	0
<i>Pseudomonas aeruginosa</i>	0	0	0	0	24	20	6	0	24	20	6	0
<i>Pseudomonas aeruginosa</i>	0	0	0	0	6	0	0	0	8	0	0	0
<i>Klebsiella pneumonia</i>	0	0	0	0	20	14	0	0	24	20	14	0

Table 3. Zone diameter of inhibition in mm of latex, and stem and leaves extracts on Bacteria.

2.3 Effects of Aveloz Latex Stem and Leaf Extracts on Fungus Isolates

Although aveloz latex had no antibacterial activity, it showed antifungal activity at high concentration against *Aspergillus*, *Mucor*, *Rhizopus* and *Candida* spp. The effects obtained were either due to the pure extract or a 1:10 dilution. The results are shown in (Table 4). The extracts obtained from the stem and leaves had limited or no activity against fungi, the results is summarized in Table 6 below.

Table 4. Diameter of inhibition zones in mm, caused by aveloz materials on fungi.

Fungus	Aveloz latex concentrations					Aveloz stem extracts concentrations				Aveloz leaves extracts concentrations			
	Origin	1:1	1:2	1:4	1:8	Origin	1: 1	1: 2	1: 4	Origin	1: 1	1: 2	1: 4
<i>Aspergillus</i>	28	20	0	0	0	6	4	0	0	6	6	0	0
<i>Mucor</i>	20	16	0	0	0	16	10	0	0	10	6	0	0
<i>Fusarium</i>	0	0	0	0	0	6	0	0	0	8	5	0	0
<i>Yeast</i>	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Rhizopus</i>	30	26	20	16	0	6	0	0	0	4	0	0	0
<i>Candida</i>	16	10	6	0	0	17	8	0	0	14	8	6	0
<i>yeast</i>	20	0	0	0	0	0	0	0	0	0	0	0	0
<i>Mold</i>	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Mold</i>	0	0	0	0	0	6	6	0	0	6	0	0	0

IV. Discussion

Our results indicated that aveloz latex suppressed and prevented the growth of mouse breast cancer cells. At high concentration, it caused cell lyses and drastically reduced cell viability at lower concentration. A study speculated that the mechanism of the antitumor activity of *euphorbia tirucalli* may be related to the regulation of granulocyte and macrophage production and expression of functional activities (4). Another study revealed that latex has induced suppressed activity on CD4+ and CD8+ T-cells, inhibited interleukin-2 and gamma- interferone production and affected immunomodulation (5). It was finding that latex taken internally caused reduction in lymphocytes number (6). Research conducted on tumor bearing mice with myelosuppression and increased number of spleen CFU-GM, latex reduced these cells (spleen colony formation), reduced prostaglandin E2 that is normally elevated in tumor bearing mice and caused reduction in tumor growth in the peritoneal cavity. This indicates the presence of antitumor activity of *euphorbia tirucalli* (4). A study on latex revealed that it enhanced the growth of EBV which led to Burkitts lymphoma (7). Literature search did not reveal many articles related to the antitumor activity of this herb. Most of the information provided in literature lacked scientific basis. No articles were detected in literature dealing with the effects of aveloz on breast cancer cells tested in this work or any other cell lines. The antibacterial and antifungal properties of latex were not investigated in literature. The only article found indicated that extracts from the stem and leaves of *Euphorbia tirucalli* have anti herpes simplex virus -2 effects (8). As indicated in the results obtained in this project, the aveloz latex gave some antifungal activity. The numbers of the fungal isolates tested were not enough to draw major conclusion. Extracts from the leaves and stems of this herb demonstrated antibacterial activity. Most of the gram positive and gram negative bacterial isolates were susceptible to this extract to a reasonable dilution (1:4). Exceptions were seen in multidrug resistant *A. baumannii*, *Pseudomonas aeruginosa*, and *Salmonella* and *Shigella* species. The drawbacks of the antimicrobial effects of this herb are the unavailability of literature discussing this issue. Therefore we are unable to compare our results with others.

V. Conclusion

Aveloz may be a promised medical plant to produce medicinal preparations for treatment different bacterial, fungal and tumor diseases, so the results of this research give informational supports to work on this plant by pharmaceutical companies' researches.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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